JOURNAL OF BACTERIOLOGY, Feb. 1980, p. 1091-1094
0021-9193/81/021094-04$02.00/0

Vol. 145, No. 2

Escherichia coli Growth Studied by Dual-Parameter Flow Cytophotometry

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The growth of *Escherichia coli* cells has been analyzed for the first time by dual-parameter flow cytophotometry, in which the deoxyribonucleic acid and protein contents of single bacteria have been measured simultaneously with an accuracy of a few percent and at a rate of 3,000 cells/s.

Flow cytophotometry is being established rapidly as a major experimental method in various fields of cell biology (5). With this technique one can measure in single cells the contents of various components, such as DNA, RNA, and proteins, with an accuracy of a few percent and at a rate of several thousand cells per second. The cells are stained with a fluorescent dye that binds specifically and quantitatively to the appropriate component. Carried in suspension by a microscopic, laminar flow of water, they are passed through a focus of exciting light, so that each cell gives rise to a pulse of fluorescence, the intensity of which is proportional to the cellular contents of the stained component. A sensitive detector transforms the fluorescence pulses into equivalent electrical pulses which are sized and stored by a multichannel pulse height analyzer. The multichannel analyzer thus accumulates a histogram of the cells with regard to their contents of the component. Much more information is obtained with dual-parameter flow cytometers which simultaneously detect two different spectral fluorescence components in separate channels. Alternatively, the excitation light scattered by the cells can be measured as the second parameter to obtain information on cell size and structure. So far, the numerous applications of flow cytometry have been limited almost exclusively to eucaryotic cells, whereas only a few single-parameter studies on procaryotes have been reported (1, 11, 12). We describe here a flow cytometric dual-parameter (DNA-light scattering) analysis of the growth of *Escherichia coli* cells.

Cells of *E. coli* K-12, strain W 3110, were grown in aerated K-glucose medium at 37°C (3). Samples were diluted 10-fold in cold 70% aqueous ethanol for fixation and stained with a combination of ethidium bromide and mithramycin (2), which is highly specific for DNA and which binds quantitatively, so that the amount of dye per cell is proportional to its content of DNA. Fixation and staining of the cells did not noticeably alter their size or shape as observed through the microscope.

By using a laboratory-built flow cytophotometer that is based on a standard fluorescence microscope (15, 16), the DNA-associated fluorescence and light scattering of the cells were recorded simultaneously, yielding three-dimensional histograms (Fig. 1). Corresponding histograms of cells with their total protein stained by fluorescein-isothiocyanate showed that within experimental accuracy there was a linear relationship between light scattering and fluorescein-isothiocyanate fluorescence, indicating that with the present instrument light scattering per cell is proportional to total cell protein, i.e., essentially to the dry mass of the cell (16). Hence, the histograms in Fig. 1 relate cellular DNA and protein.

The histogram of exponentially growing cells (Fig. 1A) has a unimodal appearance, indicating a close relationship between cellular DNA and protein. By contrast, the corresponding histogram for cells cultured in the presence of chloramphenicol (CAP) (Fig. 1B) exhibits two distinct peaks, one at twice the DNA content of the other. The two small peaks at lower light scattering values probably represent decaying cells.

Each three-dimensional histogram can be broken down to relate the parameters pairwise by projecting it onto its three different planes so as to produce a DNA histogram, a light scattering (i.e., protein) histogram and a curve relating DNA and protein (Fig. 2). The DNA histogram (Fig. 2A) of the cells treated with CAP shows more clearly that one peak is at twice the DNA content of the other.

To identify these peaks, i.e., to calibrate the fluorescence axis with regard to cellular DNA content, cells of strain E177 (*dnaA*) (from B. Bachmann) were grown for 2 h at 42°C and fixed and stained as before. This strain is defective in initiation of new rounds of DNA replication at the restrictive temperature and will end up with one full chromosome after prolonged growth at

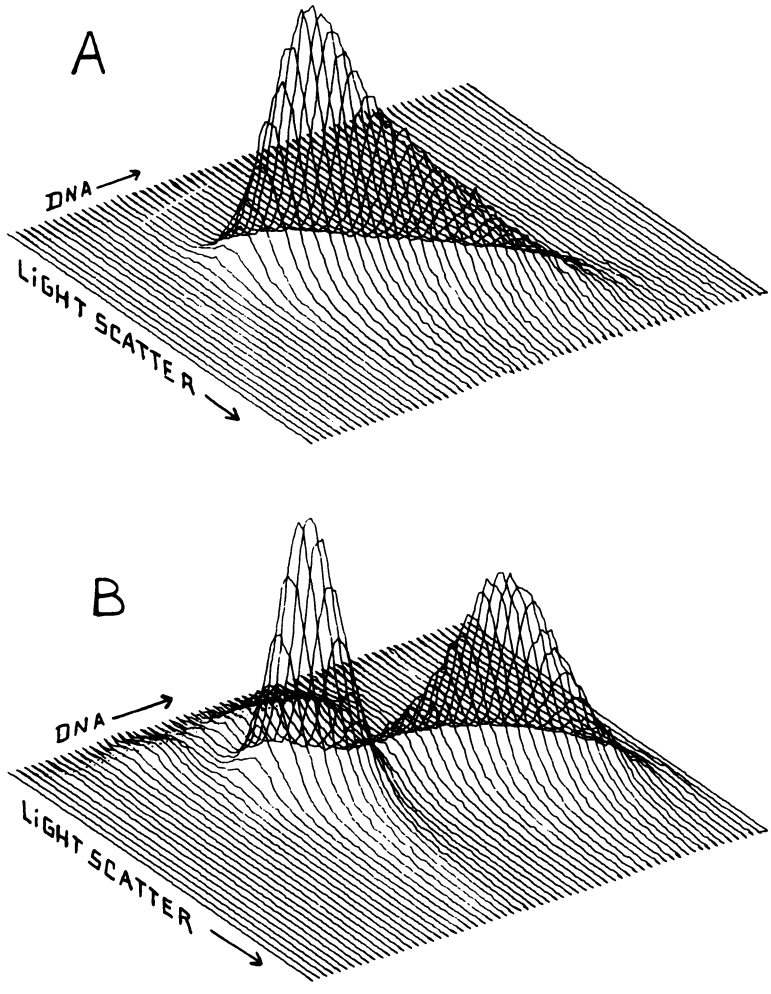

FIG. 1. *Three-dimensional DNA-light scattering histograms of E. coli harvested during exponential growth (A) and after incubation with CAP (B). CAP (50 μg/ml) was added to the exponentially growing culture which was incubated for another 4 h before harvest. Cells were fixed in ice-cold 70% aqueous ethanol, washed in Tris (pH 7.4), and suspended in 25 μg of ethidium bromide per ml in Tris, (pH 7.4) which was supplemented with an equal volume of 100 μg of mithramycin (Pfizer) per ml in 25% aqueous ethanol with 0.15 M $MgCl_2$. The histograms were recorded with a flow cytophotometer in which each cell, carried in a laminar flow of water, is passed through the focus of a fluorescence microscope at a speed of about 20 m/s. Each cell gives rise to a pulse of fluorescence which is proportional to its DNA content and a pulse of scattered light proportional to its dry mass. Photomultiplier detectors transform the light pulses into equivalent electrical ones. These pulses are measured by a dual parameter multichannel pulse height analyzer and stored in its array of 64 × 64 channels so that channel numbers are proportional to pulse heights, i.e., to cellular DNA content and dry mass, respectively. Each of the histograms represent 4.2×10^5 cells, which were recorded in about 3.5 min.*

42°C. The histogram of these cells (data not shown) exhibited one sharp peak at half the fluorescence intensity of the narrow peak in Fig. 1B. Thus, peaks were observed at fluorescence intensities corresponding to 1X, 2X, and 4X a certain DNA content. It seems an obvious interpretation that this DNA content is that of a single chromosome. Accordingly, the two peaks in Fig. 1B and 2A (CAP) represent cells with two and four full chromosomes.

This interpretation is further supported when the present data are compared with the widely accepted model for the proliferation kinetics of *E. coli* (4, 7) which is based on data obtained by various chemical assays. Knowing the cell doubling time, DNA replication time, and time between end of DNA replication and cell division, one may calculate the average DNA content per

cell from this model. As seen from Table 1, such values for two cultures with widely different cell doubling times are in close agreement with those

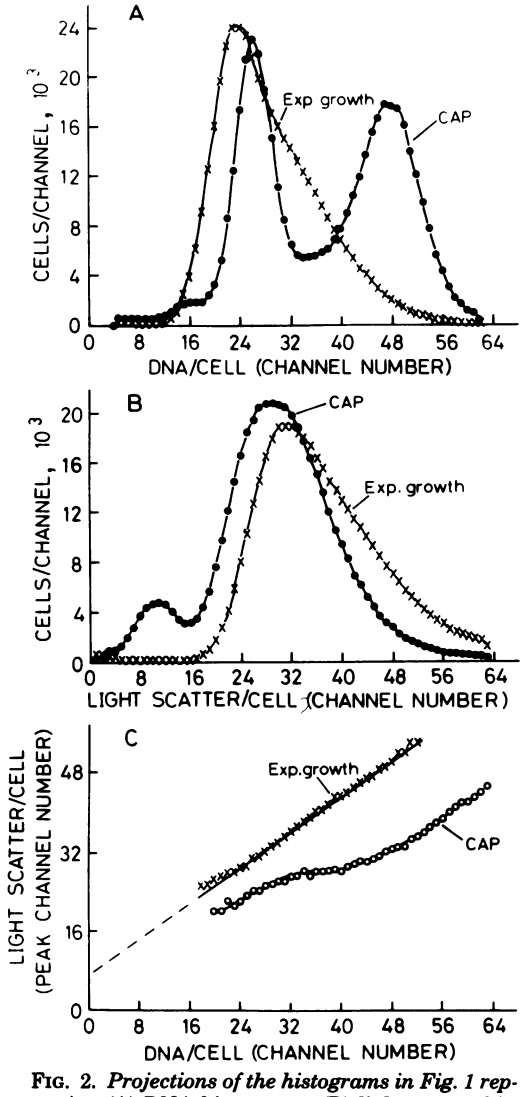

FIG. 2. *Projections of the histograms in Fig. 1 representing (A) DNA histograms, (B) light scatter histograms, and (C) the correlation between DNA and light scattering. The latter curves trace the ridge of the histograms in Fig. 1, i.e., the light scattering peak channel number as a function of the DNA channel number. The two prominent peaks in the DNA histogram of CAP-treated cells represent cells with two and four complete chromosomes, respectively. About 44% of the cells are in the two-chromosome peak. The widths of the two peaks correspond to a relative standard deviation of CV = 10%. Instrumental resolution was 5%. The average DNA content of exponentially growing cells corresponded to 2.1 chromosomes per cell, in accordance with a cell doubling time of 37 min.*

TABLE 1. *Theoretical and experimental values of average cellular DNA content of E. coli growing exponentially at two different growth rates*

Medium	Doubling time[b] (min)	Cellular DNA	
		Theoretical[c]	Experimental[d]
K-glucose	30	2.61	2.4 ± 0.2
DL-alanine[a]	92	1.35	1.4 ± 0.1

[a] Same as K-glucose, except with Casamino Acids and glucose replaced by 2 mg of DL-alanine per ml.

[b] Cell doubling time measured by counting with a Coulter Counter at 20-min intervals.

[c] Average cellular DNA content calculated from theoretical model (4, 7), assuming a DNA replication time of 40 min and the time from the end of DNA replication to cell division equal to 20 min, in accordance with literature values (4).

[d] Values derived from DNA histograms.

derived from DNA histograms on the assumption of the above interpretation of the histogram peaks.

The fluorescence intensity of cells with two chromosomes was 465 times smaller than that of human diploid lymphocytes stained by the same procedure. In comparison the DNA contents of these cells differ by a factor of 700. The main reason for the discrepancy is probably that parts of the human DNA are so densely covered by histones that the dye is prevented from entering.

The histogram of Fig. 2A shows that in the presence of CAP, which inhibits protein synthesis, the cells were able to complete chromosomes already initiated. As indicated by the lack of cells with one chromosome, cell division did not occur. This is in accordance with the observation that protein synthesis is required for cell division (6, 14). Initiation of chromosome replication was not completely inhibited, but strongly reduced, in accordance with other reports (8–10). A comparison of the number of cells with two chromosomes or less in the DNA histograms of cells harvested when CAP was administered with that of cells grown with CAP for 4 h showed that about 20% of the cells escaped the two-chromosome peak in the presence of CAP. Histograms obtained at shorter intervals showed that most of this initiation of chromosome replication took place during the first 0.5 h after CAP was given. The latter histograms also showed that it took about 90 min for the two-chromosome peak to develop completely and some 4 h for the four-chromosome peak (17) to develop, as compared with the 40 min required for a round of DNA replication in the absence of CAP (4). Hence, the rate of DNA synthesis was

greatly reduced in the presence of CAP and more so for cells replicating two chromosomes than for cells replicating one. A reduced rate of DNA synthesis in the presence of CAP has been reported earlier (13).

As shown in Fig. 2B the light scattering histogram of cells harvested after 4 h in the presence of CAP was shifted towards lower values by about 15% as compared with that of cells harvested when CAP was given. Hence, the average protein content per cell appeared to decrease in the presence of CAP. This confirms the inhibitory effect of CAP on protein synthesis and demonstrates that DNA replication continued—although at a reduced rate—in cells that actually lost protein. A close analysis of these histograms shows that the rate of protein loss per cell in absolute units was proportional to total cell protein.

The curves relating light scattering, i.e., cellular protein, and DNA (Fig. 2C) demonstrate a linear relationship between total cell protein and DNA throughout the cell cycle of exponentially growing cells. In this respect *E. coli* are quite different from eucaryotic cells which have a substantial portion of their protein synthesis in the G1 and G2 phases, where little DNA synthesis takes place. In the corresponding curve for the CAP-treated cells, the upper part of the curve, representing cells with a DNA content corresponding to more than two complete chromosomes, is shifted laterally toward higher DNA content as expected when DNA synthesis occurs without a concurrent increase of cell protein.

The present data, which were recorded altogether in about 7 min, exemplify the degree of detail, precision, and speed by which bacteria can be studied by flow cytophotometry. By recording information on single cells in large numbers, this method makes it possible to distinguish subpopulations of cells and to discern details which cannot be detected by other methods. For bacteriological purposes it seems specially suited for studies of growth kinetics. For example, assessment of drug response, including which part of cell cycle is affected, can be carried out in a few hours.

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